

Clotting factors secreted by monocytes and macrophages

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CLOTTING FACTORS SECRETED BY MONOCYTES AND MACROPHAGES: ANALYTICAL CONSIDERATIONS.

Maria C.E. van Dam-Mieras, Annemarie D. Muller, Wijgert A. van Deijk,
H. Coenraad Hemker,
Department of Biochemistry, Limburg University, Maastricht, The Netherlands.

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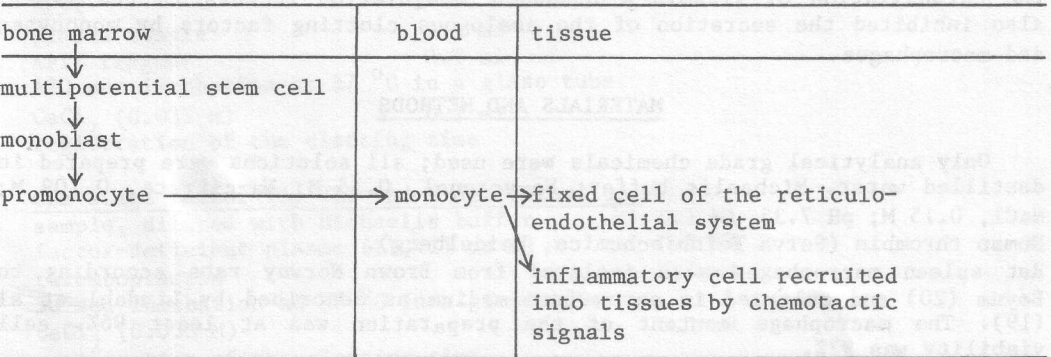
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ABSTRACT

The secretion of clotting factors by rat spleen macrophages and human peripheral blood monocytes has been studied. The results show that the amount of clotting factors measured depends critically upon the characteristics of the assay system used. The presence of warfarin, salicylic acid or thrombin in the culture medium is shown to decrease the vitamin K dependent clotting factor activity in the supernatant after in vitro culture of rat spleen macrophages and human peripheral blood monocytes.

INTRODUCTION

The course of life of cells belonging to the monocyte-macrophage series is given schematically below:



Key words: monocytes, macrophages, clotting factors, clotting assays.

Cells of the monocyte-macrophage series can be triggered by a variety of stimuli and the responses observed are determined by the stimuli used. Among the responses observed are: phagocytosis, bactericidal activity, tumoricidal activity and secretion of biologically active products into the extracellular surroundings. Among the secreted products are enzymes that can activate the plasma proteins of the coagulation-, fibrinolytic- and complement pathways. These enzymes form a link between the hemostatic and immune systems (1-3).

Cells of the monocyte-macrophage series can also expose procoagulant activity on their surface after exposure to a variety of stimuli including bacterial endotoxins, antigen/antibody complexes, mitogenic lectins and the proteolytic products of complement (2,4-10).

Different types of cellular procoagulant activity have been described:

1. tissue factor-like activity (2,4-7,10,11)
2. prothrombin activating enzyme(s) (2,6,12,16)
3. activated clotting factor VII (13,14,15)

Østerud et al. (17,18) and Lindahl et al. (19) have shown that unstimulated murine peritoneal macrophages cultured in serum free medium secrete the coagulation factors II,V,VII,IX and X into the culture medium and do not expose tissue factor on their surface under these conditions. If, however, the macrophages have been stimulated during their isolation by endotoxin (in the presence of T-lymphocytes), thromboplastin is generated on the cellular surface and the coagulation factors secreted into the medium occur in their activated forms. The thrombin level in the medium is reduced under these conditions.

The possibility, that a combination of the capacity of monocytes and macrophages to generate and expose tissue factor complex components (apoprotein and phospholipid) on the cellular surface and their capacity to synthesize and secrete clotting factors into the surrounding medium might explain the different procoagulant phenotypes that have been described, has not received much attention in the literature as far as we know.

As the "cellular procoagulant activity" of monocytes and macrophages forms a link between immunology and coagulation, the possible role of the "cellular procoagulant activity" in the defense against infection and malignancy and the possibility to use it as a parameter to follow pathological processes is very interesting. We therefore decided to carefully characterize the different procoagulant factors secreted by rat spleen macrophages and human peripheral blood monocytes and to set up species specific test systems. We further investigated if compounds like warfarin and aspirin, known to inhibit the carboxylation of vitamin K-dependent coagulation factors in the liver, also inhibited the secretion of the analogous clotting factors by monocytes and macrophages.

MATERIALS AND METHODS

Only analytical grade chemicals were used; all solutions were prepared in distilled water. Michaelis buffer: Na-veronal, 0.15 M; Na-citrate, 0.109 M; NaCl, 0.15 M; pH 7.35.

Human thrombin (Serva Feinbiochemica, Heidelberg).

Rat spleen macrophages were isolated from Brown Norway rats according to Bøyum (20) and cultured in serum free medium as described by Lindahl et al (19). The macrophage content of the preparation was at least 96%. cell viability was 97%.

Human peripheral blood monocytes were isolated and cultured as described by Bøyum (20) using ficoll paque (Pharmacia). The monocyte content of the preparation was at least 95%; cell viability was 96%.

Analysis of cell composition was performed on cytocentrifuge preparations stained with Giemsa or non specific esterase (21). At least 200 cells were counted per slide. Viability was assessed by trypan blue exclusion.

Culture medium: RPMI 1640 medium (Flow laboratories), penicilline (Gibco, final concentration 100 IU/ml), streptomycine (Gibco, final concentration 100 IU/ml), transferrine (Behringwerke, final concentration 10 μ g/ml), albumin (Behringwerke, final concentration 0.4 mg/ml). In several experiments this culture medium was supplemented with additional compounds; the exact composition of the culture medium will be specified in the description of these experiments.

Culture medium and reagents were free from endotoxin contamination as determined by a chromogenic substrate endotoxin assay (KabiVitrum Diagnostica)(22).

The in vitro effect of phospholipase C on the procoagulant activity generated by monocytes or macrophages was tested as follows (23,24): a mixture of 0.1 ml citrated plasma, 0.1 ml sample and 0.1 ml phospholipase C (1 μ g/ml) was incubated for 3 min at 37 °C, then the clotting was initiated by the addition of 0.1 ml CaCl_2 (0.33 M). The clotting time observed was compared to that obtained when carrying out the same test with 0.1 ml Michaelis buffer instead of phospholipase C.

Rat standard plasma was obtained by pooling equal amounts of platelet free plasma from 30 Brown Norway rats (15 males and 15 females) and was stored in small portions at -20 °C until use (25). Rat brain thromboplastin (Brown Norway rats) was prepared as described before (25). Rat artificial factor II reagent was prepared according to Koller et al. (26) using oxalated rat plasma and rat serum.

Human standard plasma was obtained by pooling equal amounts of platelet free plasma from at least 30 healthy individuals (15 males and 15 females, average age 30 years) and was stored in small portions at -20 °C prior to use (25). Human brain thromboplastin was prepared as described before (25). Human artificial factor II reagent was prepared according to Koller et al. (26) using human oxalated plasma and human serum.

For the determination of the clotting factors VII, IX and X human congenital factor deficient reagents were used. APTT-reagent was obtained from Dade.

thromboplastin time:

plasma sample	0.1 ml
thromboplastin	0.1 ml
30 sec incubation at 37 °C in a glass tube	
CaCl_2 (0.033 M)	0.1 ml
registration of the clotting time	

APTT

plasma sample	0.1 ml
APTT reagent	0.1 ml
120 sec incubation at 37 °C in a glass tube	
CaCl_2 (0.033 M)	0.1 ml
registration of the clotting time	

One stage factor II, VII and X determination:

sample, diluted with Michaelis buffer	0.1 ml
factor-deficient plasma (II,VII or X)	0.1 ml
thromboplastin	0.1 ml
30 sec incubation at 37 °C in a glass tube	
CaCl_2 (0.033 M)	0.1 ml
registration of the clotting time	

One stage factor IX determination:

sample, diluted with Michaelis buffer	0.1 ml
factor IX-deficient plasma	0.1 ml
APTT reagent	0.1 ml
300 sec incubation at 37 °C in a glass tube	
CaCl ₂ (0.033 M)	0.1 ml
registration of the clotting time	

RESULTS

In a one stage clotting test the level of the rate-determining factor is P estimated from its effect on the overall coagulation cascade and is expressed in % of standard plasma by comparing the coagulation time observed with the sample to those of a series of dilutions of a standard plasma (= pooled normal plasma). It can be expected that for such a bioassay the result of the measurement will depend upon the composition of the assay mixture, in other words: standardization is very important.

It is well-known that the interaction between tissue thromboplastin and coagulation factor VII exhibits a rather high degree of species specificity (27-29). Species specificity may also be relevant in other steps of the coagulation cascade but, the effect seems to be much less pronounced there than in the thromboplastin-factor VII combination step. For the reasons given above, it is strongly advisable to assay clotting factors from a given species in a test system in which all components are derived from that specific species. However, if there are no congenital or artificial factor deficient reagents available for the species studied, a good approximation is:

1. to use thromboplastin of the species studied in factor VII determinations and thromboplastin from the same source as the factor deficient reagents in the determination of the other factors. (In this setup the factor VII and the thromboplastin that have to combine will always originate from the same source)
2. to use pooled normal plasma of the species studied as standard plasma for the construction of the reference curve.

This principle is illustrated in table I.

TABLE I

The Relation between the Thromboplastin Time Observed and the Origin of Thromboplastin and Standard Plasma.

standard plasma source	thromboplastin source	coagulation time
human	human	14.5 sec
human	rat	39.0 sec
rat	rat	43.1 sec
rat	human	16.7 sec

It can be seen in this table that the coagulation time obtained is a function of both the source of thromboplastin used to initiate the reaction and the source of the standard plasma used to construct the reference curve. The principle illustrated above for the overall clotting reaction is also valid for the determination of the individual clotting factors. The dependence of the observed clotting time on the thromboplastin- and standard

plasma source will of course have its implications for the translation of a measured clotting time into a clotting factor level.

The principles outlined above must be applied consequently. We therefore proceed as follows when measuring clotting factors secreted into the culture medium:

1. Construction of the reference curve: pooled normal plasma of the species studied is diluted 1:1 with culture medium; the solution obtained is diluted further with Michaelis buffer.
2. Measurements: Culture medium samples are diluted 1:1 with Michaelis buffer prior to measurement.

The analytical principles outlined above are clearly illustrated in the tables II and III in which the amount of clotting factors secreted into the culture medium by respectively rat spleen macrophages and human peripheral blood monocytes are given.

TABLE II

Coagulation Factors Secreted by Rat Spleen Macrophages

Incub. time	%II			%VII			%IX			%X		
	Lit	hum	rat	Lit	hum	rat	Lit	hum	rat	Lit	hum	rat
24 h	4-7	2	8-14	8-12	8-17	4-6	5-8	6-8	6-7	7-11	2-4	6-8

macrophages were cultured in serum free medium supplement with vitamin K (0.1 Mg/ml) for 24 hours; n=8.

Lit: ref 14; hum: tested in "human system"; rat: tested in "rat system".

TABLE III

Coagulation Factors Secreted by Human Peripheral Blood Monocytes

incub. time	%II		%VII		%IX		%X	
	hum	rat	hum	rat	hum	rat	hum	rat
24 h	8	<0.5	5	<0.5	4	2.5	2.5	<0.5

monocytes were cultured in serum free medium supplement with vitamin K (0.1 Mg/ml) for 24 hours; n=4.

hum: tested in "human system"; rat: tested in "rat system".

The amount of clotting factors secreted after 16 hours culture in the presence of vitamin K alone were very similar to those found after 24 hours in culture.

We also investigated the influence of different culture conditions upon the amount of clotting factors secreted into the culture medium by rat spleen macrophages. Warfarin and salicylic acid were added because they are known to

TABLE IV

Coagulation Factors Secreted by Rat Spleen Macrophages Cultured under Different Conditions for 24 Hours

addition to the culture medium	%II	%VII	%IX	%X
vitamin K	12	5	6	7
vitamin K + warfarin	0.8	3	<0.5	<0.5
vitamin K + salicylic acid	1	3	<0.5	0.8
vitamin K + thrombin	0.8	2.5	<0.5	1.5
warfarin	<0.5	<0.5	<0.5	<0.5
salicylic acid	1	4	<0.5	0.8
thrombin	2	10	<0.5	1.0

Rat spleen macrophages were cultured in serum free medium supplemented with the substances indicated. The final concentrations were: vitamin K: 0.1 Mg/ml; warfarin: 12.5 Mg/ml; salicylic acid: 20 Mg/ml; thrombin: 0.05 NIH Units/ml. The clotting test was carried out in a "rat test system".

inhibit the carboxylation of vitamin-K dependent clotting factors in the liver (30,31). We also carried out an experiment in which a trace amount of thrombin was added to the culture medium because thrombin not only has a special position in the coagulation cascade, but it also seems to have other important biological functions such as the induction of a procoagulant surface in thrombocytes (32), the attraction of phagocytosing cells towards the site of injury (33) and the induction of mitogenic and other cellular activities (34-36).

In table V the same type of experiment carried out with human peripheral blood monocytes is described; the results are very similar to those obtained with rat spleen macrophages.

TABLE V

Coagulation Factors Secreted by Human Peripheral Blood Monocytes Cultured under Different Conditions for 24 Hours.

addition to the culture medium	%II	%VII	%IX	%X
vitamin K	8	5	4	2.5
warfarin	<0.5	<0.5	<0.5	<0.5
thrombin	10	2	<0.5	<0.5

Human peripheral blood monocytes were cultured in serum free medium supplemented with the substances indicated. The final concentrations were: vitamin K: 0.1 Mg/ml; warfarin; 12.5 Mg/ml; thrombin: 0.05 NIH Units/ml. The clotting tests were carried out in a "human test system".

Monocytes and macrophages have been shown to expose thromboplastin on the cellular surface upon stimulation. If thromboplastin like material would be released into the supernatant during the 24 hours in vitro culture, this process could be expected to interfere with the factor VII determination. We

therefore investigated the effect of phospholipase C treatment on culture medium samples. It was shown that the culture medium itself was not sensitive to phospholipase C treatment. However, after 24 h in vitro culture of monocytes or macrophages phospholipase C treatment of the culture supernatant caused a prolongation of the clotting time of this fraction.

DISCUSSION

In order to properly characterize the procoagulant activity generated by monocytes and macrophages under different experimental conditions a characterization of the test system per se is necessary. It is clear from the presented results that careful standardization of the clotting assays is necessary. It will be evident that the translation of an observed clotting time into a clotting factor level is dependent on the thromboplastin used to initiate the reaction and the standard plasma used to construct the reference curve. We therefore state that, for the determination of overall clotting activity as well as for single factor determinations, the best experimental approach is to measure human peripheral blood monocytes in a "human test system" and rat spleen macrophages in a "rat test system". By a "human test system" we mean a test system in which we use human thromboplastin, human standard plasma and human congenital or artificial factor deficient reagents. In principle the "rat test system" should be constructed analogously. However, for the rat no congenital factor deficient reagents are available, and, although we have been able to prepare a rat artificial factor II deficient reagent, the preparation of other rat artificial factor deficient reagents was impossible because of the limited amounts of rat plasma available. We therefore were obliged to use the following experimental design for the "rat test system":

- in the factor II determination all components of the test system are from a rat source
- in the factor VII determination the standard plasma and the thromboplastin are from a rat source, the congenital factor VII deficient reagent is from human origin
- in the factor X determination the standard plasma is from a rat source and both the thromboplastin and the congenital factor X deficient reagent are from human origin.

This experimental approach ensures that in all assays the proper thromboplastin-factor VII combination is present and that the unknown sample is always compared to a standard from the same source.

For the determination of clotting factors in the culture medium the composition of standard and test samples was matched by the dilution of pooled normal plasma with culture medium prior to further dilution with Michaelis buffer in the construction of the reference curve and by the dilution of the unknown sample with Michaelis buffer prior to testing.

The importance of the test system composition is evident from the tables II and III in which the amount of clotting factors secreted into the serum free culture medium by respectively rat spleen macrophages and human peripheral blood monocytes during a 24 hours in vitro culture is given. The literature values in table II are taken from Østerud et al. (17). We have not been able to compare the amount of clotting factors secreted into the culture medium by human peripheral blood monocytes to literature values, because they have not been published before. Lindahl et al (19) report that, according to their preliminary results, human peripheral blood monocytes do not secrete coagulation factors into the culture medium. As it is evident from table III that the measured amount of clotting factors secreted into the culture medium by human peripheral blood monocytes is critically dependent upon the assay system used, we wonder if this discrepancy between our findings and those of Lindahl et al. could be explained by analytical problems.

The reduction of the amount of vitamin K-dependent clotting factors secreted into the culture medium by rat spleen macrophages and human peripheral blood monocytes upon supplementation of the culture medium with warfarin and aspirin is exactly what could be expected on account of the influence of these substances upon the synthesis of the analogous clotting factors in the liver (30,31) and was also reported by Østerud et al. (17). However, as can be seen in the tables IV and V, the factor VII level is not reduced to the same extent upon the addition of warfarin and aspirin to the culture medium as that of the other clotting factors. We have no straightforward explanation for this effect but we can imagine that, if some tissue thromboplastin like material is detached from the cellular membrane this material can form a complex with the factor VII molecules present in the culture medium, thereby potentiating the factor VII biological activity. As a result of this process the apparent factor VII concentration as determined by a one stage factor VII test will be too high. The finding that phospholipase C treatment of culture supernatants causes a prolongation of the clotting time of this fraction also points in this direction. We therefore would like to conclude that monocytes and macrophages "loose" phospholipase C sensitive material into the culture medium during a 24 hours in vitro culture. At present we have no idea if the occurrence of phospholipase C sensitive material in the culture medium has a physiological meaning, it can also be an artefact produced during the 24 hours in vitro culture. In the in vivo situation this question could be rephrased as: is the transfer of clotting activity from the monocyte and macrophage surfaces into the surrounding medium physiologically meaningful or is it merely an unwanted side-effect caused by damaged cells?

The low amount of clotting factors found in the culture medium after cultivation of the cells in the presence of trace amounts of human thrombin is rather surprising and could tentatively be explained by a thrombin-dependent activation of the secreted clotting factors followed by a process inhibitory to active serine proteases, like for instance binding to a cell surface receptor for active serine proteases (37,38). Again the relatively high residual level of factor VII under these conditions could be explained by the presence of thromboplastin like material in the culture medium. Factor VII bound to this material can be expected to show less affinity for inhibitory compounds. This interesting effect of thrombin on monocytes and macrophages will be further investigated.

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